Identification of Arabidopsis *rat* Mutants

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Limited knowledge currently exists regarding the roles of plant genes and proteins in the *Agrobacterium tumefaciens*-mediated transformation process. To understand the host contribution to transformation, we carried out root-based transformation assays to identify Arabidopsis mutants that are resistant to *Agrobacterium* transformation (*rat* mutants). To date, we have identified 126 *rat* mutants by screening libraries of T-DNA insertion mutants and by using various "reverse genetic" approaches. These mutants disrupt expression of genes of numerous categories, including chromatin structural and remodeling genes, and genes encoding proteins implicated in nuclear targeting, cell wall structure and metabolism, cytoskeleton structure and function, and signal transduction. Here, we present an update on the identification and characterization of these *rat* mutants.

Agrobacterium tumefaciens-mediated genetic transformation is widely used to generate transgenic plants of many economically important plant species, but there remain many challenges for applying this technique to numerous recalcitrant species and elite varieties of agronomic and horticultural importance. These include major cereal crops (maize [*Zea mays*], rice [*Oryza sativa*], wheat [*Triticum aestivum*], barley (*Hordeum vulgare*), oat (*Avena sativa*), etc.), legumes (soybean [*Glycine max*], common bean [*Phaseolus vulgaris*], and pea [*Pisum sativum*]), cotton (*Gossypium hirsutum*), fruit, nut, and ornamental trees, and trees used for timber and pulp production (van Wordragen and Dons, 1992; Hansen and Wright, 1999; Pena and Seguin, 2001). The molecular and genetic events within *A. tumefaciens* leading to plant transformation are reasonably well understood. However, we currently have very limited knowledge of the roles that plant genes and proteins play during this process (for reviews, see Gelvin, 2000, 2003a; Zupan et al., 2000; Tzfira and Citovsky, 2002; Wu and Hohn, 2003). Further investigation of the functions of host genes and manipulation of their expression may lay a foundation for the improvement of transformation of recalcitrant plants (Gelvin, 2003b).

The *A. tumefaciens*-mediated transformation process results from a complex interaction between the host and the bacterium. The events that occur within the bacterium include the perception of phenolic and sugar signals, induction and expression of the *vir* (virulence) genes, processing of the T-(transferred) DNA from the tumor-inducing plasmid, and export of the T strand (the single-stranded processed form of T-DNA) and virulence proteins from the bacterium using a type IV secretion system encoded by the *virB* and *virD4* genes (see e.g. Christie and Vogel,

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2000). Events involving the plant include attachment of the bacterium to the plant surface, transfer of the T-DNA and virulence proteins through the plant cell wall and plasma membrane to the cytoplasm, cytoplasmic trafficking and nuclear targeting of T-strand/protein complexes, T-DNA integration into the host genome, and the resulting expression of T-DNA-encoded genes (Fig. 1). The ultimate outcome of this complex process is the horizontal transfer of genetic information from *A. tumefaciens* to the plant genome.

One way to dissect the contribution of host factors to the *A. tumefaciens*-mediated plant transformation process is to isolate plant mutants with altered transformation properties and to identify the host genes responsible for the corresponding phenotypes. Therefore, we developed root-based transformation assays (Nam et al., 1997, 1999) because roots are a major natural transformation target for this soil bacterium. Although our initial studies entailed assessing differences in transformation susceptibility among Arabidopsis ecotypes (Nam et al., 1997), we soon focused upon screening Arabidopsis T-DNA insertion lines for plants that are resistant to *Agrobacterium* transformation (*rat* mutants; Nam et al., 1999). One advantage of screening libraries of T-DNA insertion lines is the relative ease of recovering plant DNA junction sequences at the T-DNA insertion site using plasmid rescue or thermal asymmetric interlaced PCR techniques (Liu et al., 1995). As part of a National Science Foundation-funded plant genome project, we screened, and continue to screen, several T-DNA disruption libraries for *rat* mutants. To date, we have identified more than 100 such mutants from approximately 16,500 independent T-DNA insertion lines. In addition, we have utilized several "reverse genetic" approaches to identify specific genes that are involved in the *A. tumefaciens*-mediated transformation process. These include PCR- and computer-based approaches to identify T-DNA insertions in "target" genes suspected to be involved in transformation and the use of antisense and RNAi technologies to decrease expression of "target" genes. In addition, we are currently screening Arabidopsis T-DNA activation-tagged libraries for lines that are

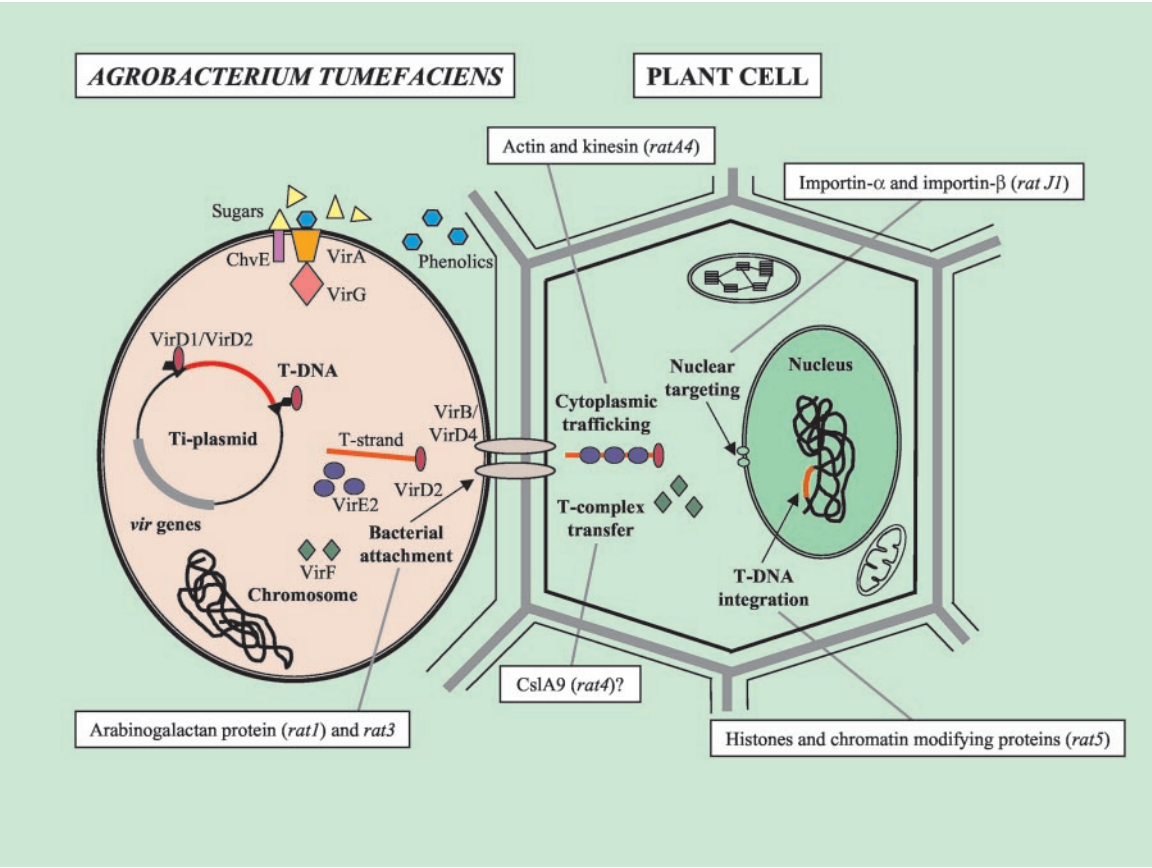

Figure 1. Schematic representation of the process of *A. tumefaciens*-mediated transformation. Phenolic and sugar molecules from wounded plant cells trigger in the bacterium a series of events resulting in the processing of the T-DNA by the VirD1/VirD2 endonuclease and the subsequent transfer of the VirD2/T-strand complex, along with VirE2 and VirF proteins, from the bacterium through the VirB/VirD4 type IV secretion system. Key events in the plant cell include bacterial attachment, T-complex and Vir protein transfer, cytoplasmic trafficking of the T-complex, nuclear targeting, and T-DNA integration. Some of the plant genes necessary for these processes are depicted by representative *rat* mutants.

hyper-susceptible to *Agrobacterium* transformation (*hat* mutants).

To date, we have identified 126 *rat* mutants and have recovered, from those generated by T-DNA insertion, numerous T-DNA/plant DNA junctions. Based on the putative functions of the encoded proteins and the various steps in the transformation process described above, we have tentatively organized these genes into several functional groups. These include cell wall metabolism and structural genes, cytoskeleton genes, genes whose products may play a role in nuclear targeting, chromatin structural and remodeling genes, and genes whose products are involved in signal transduction and housekeeping processes. The functions of the various mutated genes collectively could be involved in all steps of *A. tumefaciens*-mediated transformation, including bacterial attachment, T-DNA and virulence protein transfer, cytoplasmic trafficking and nuclear targeting of the T-complex, and T-DNA integration and expression. We were able to complement all but one of 14 selected mutants by introduction of the corresponding wild-type gene into the homozygous mutant line. Here, we present an update on the *rat* mutants that we have identified.

RESULTS AND DISCUSSION

rat Mutant Assays

We developed three root-based transformation assays to determine whether a particular T-DNA insertion, antisense, or RNAi Arabidopsis line is a *rat* mutant. The first assay measures crown gall tumorigenesis at the cut ends of root segments. We classified the morphologies of the tumors into four categories: large green leafy teratomas, small green amorphous, large yellow, and small yellow-white tumors. Generally, wild-type Arabidopsis plants fully develop tumors 4 to 5 weeks after inoculation with *A. tumefaciens* A208, although tumors can be seen with the aid of a microscope as early as 2 weeks after inoculation. The majority of tumors developing on ecotype Wassilewskija (Ws)-2 (Arabidopsis Biological Resource Center [ABRC] no. CS2360) are generally green (Fig. 2A). Tumors developing on ecotypes Columbia-0 (ABRC no. CS60000) and Columbia-7 (ABRC no. CS3731) are generally amorphous and yellow, although upon extended periods of incubation (6–8 weeks), green teratomas can occasionally develop on these ecotypes. When we score transformation, we count as positive a root segment containing any morphology of tumor. However, plants that are more susceptible to transformation respond with larger and greener tumors than do plants with decreased susceptibility. Therefore, when determining whether a particular mutant is a *rat* mutant, one must consider not only the percentage of root segments that develop tumors but also the size and morphology of the tumors. Because crown gall tumors represent a long-term response of plants to the overproduction of phytohormones directed by T-DNA-encoded genes (Weiler and Schroder, 1987), this assay measures stable transformation of the root segments. However, it is possible that plants can be stably transformed but not develop crown gall disease if the plant were a hormone response mutant (e.g. Lincoln et al., 1992). Therefore, we utilized a second screen for stable transformation: development of antibiotic resistance or herbicide tolerance encoded by a resistance gene on the T-DNA (Fig. 2A). Plant mutants that show altered susceptibility to stable transformation can be blocked at any step of the transformation process.

Plants can be transiently but not stably transformed by *A. tumefaciens* if the T-DNA reaches the nucleus and is converted to a double-stranded transcription-competent form, but the T-DNA does not integrate into the plant genome (Nam et al., 1997; Mysore et al., 1998). Thus, we developed an assay that would suggest whether a *rat* mutant were specifically T-DNA integration deficient (Fig. 2B). If roots of a particular *rat* mutant were able to express a high level of β-glucuronidase (GUS) activity 2 to 6 d after inoculation, this would indicate efficient transient transformation independent of the process of T-DNA integration. Using these sets of assays, we have confirmed biochemically that Arabidopsis ecotype UE-1 and *rat5* are integration deficient (Nam et al., 1997; Mysore et al., 2000b).

During the course of our studies, we have attempted transformation of numerous *rat* mutants using either a flower vacuum infiltration or a flower dip method (Clough and Bent, 1998). With the exception of the mutant *rad5*, the transformation of all of these mutants was as efficient as was the transformation of their respective wild-type parental ecotypes (Mysore et al., 2000a; data not shown). *rat* mutants that can be efficiently transformed by a flower dip protocol include mutants with disruptions in genes putatively involved in cell wall/membrane synthesis or function (*rat1*, *rat3*, *rat4*, and *uta1*), chromatin proteins (*rat5*, *ratT17*, *atrx1*, *ratJ7*, *HAT4*, *HAT6*, and *HDA1*), proteins involved in nuclear targeting (*ratJ1* and importin α-7), cytoskeletal proteins (*act2-1*, *act7-1*, and *act7-4*), proteins involved in transcription and signal transduction (*rat17* [*cpc*], *ratA2* [*rcn1*], *ratT5*, and *ratT8*), and unidentified or unknown proteins (*rat9*, *rat14*, *rat15*, *rat18*, *rat20*, *rat21*, *ratT16*, *ratH1*, and *ratT16*). These results are consistent with our earlier observations (Mysore et al., 2000a) and further suggest that the efficiency of transformation may depend upon the target tissue (Yi et al., 2002). Table I lists our current collection of *rat* mutants.

rat Mutants from T-DNA Insertion Libraries

We have screened and continue to screen mutagenized Arabidopsis plants from three T-DNA insertion libraries for the rat phenotype. These include

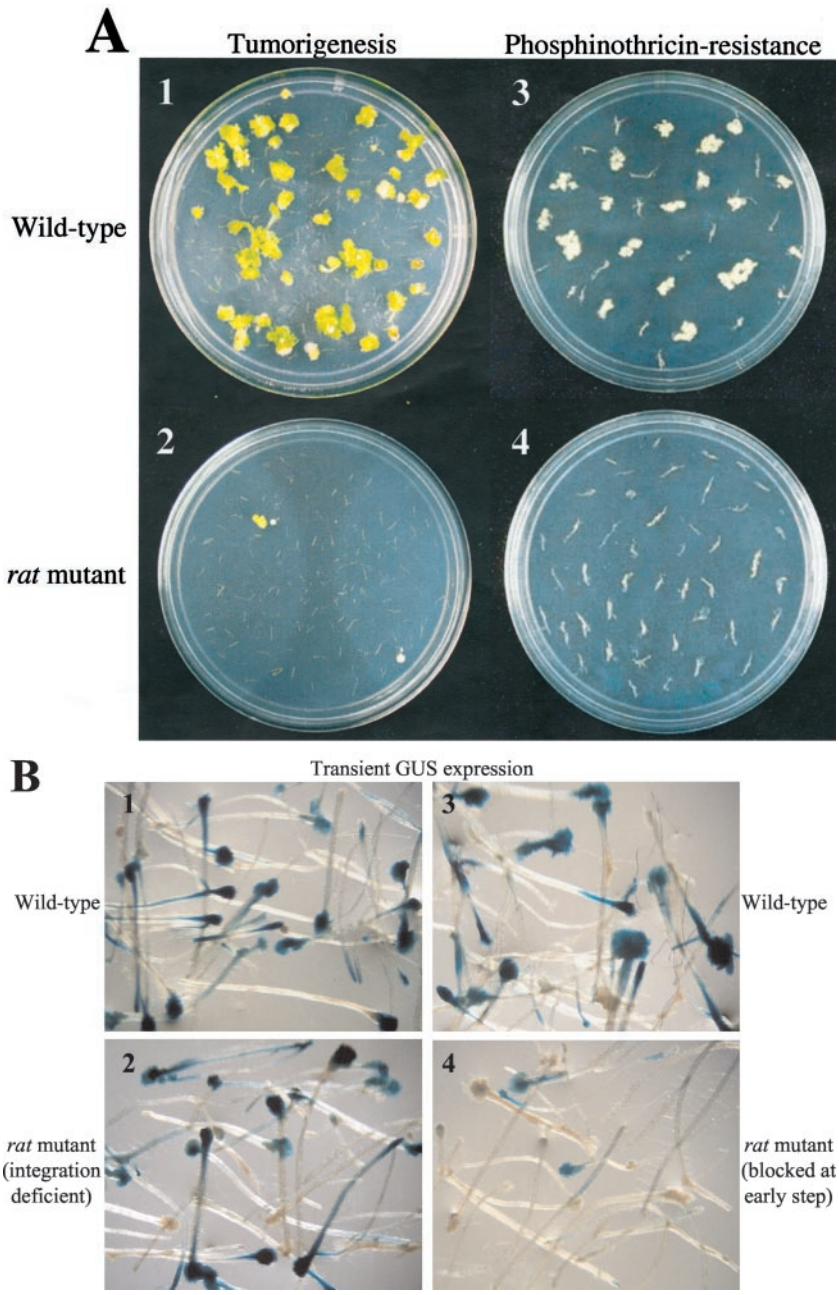

Figure 2. Wild-type and *rat* mutant phenotypes. A, Stable transformation phenotypes of crown gall tumorigenesis (1 and 2) and ppt resistance (3 and 4) on cut root segments 4 weeks after inoculation. Wild-type ecotype Ws (1 and 3) and typical *rat* mutants (2 and 4) are shown. B, Transient transformation phenotype of GUS expression 4 d after inoculation of cut root segments. Wild-type ecotype Ws (1 and 3), a *rat* mutant deficient in the step of T-DNA integration (2), and a *rat* mutant deficient in an early transformation step (4) are shown after staining with 5-bromo-4-chloro-3-indolyl glucuronide.

the Feldmann collection of 6,500 mutants (ABRC no. CS6502), the Institut National de la Recherche Agronomique (Versailles, France) collection of 3,900 mutants (ABRC nos. CS5455 and CS5600), and an 80,000-member collection of T-DNA insertion mutants generated in the laboratory of Dr. Ray Bressan (Purdue University, West Lafayette, IN). In addition, we have searched the SIGnAL TDNA-Express site (http://signal.salk.edu/cgi-bin/tdnaexpress) to identify T-DNA insertions in specific genes of interest. Consistent with an earlier report (Nam et al., 1999), approximately 0.7% of the 16,500 independent lines screened displayed a rat phenotype, suggesting that there may be as many as 200 Arabidopsis genes involved in the *A. tumefaciens*-mediated plant transformation process.

We have conducted genetic analyses of several *rat* mutants (Nam et al., 1999; Mysore et al., 2000b; data not shown). We successfully complemented all but one (*ratT8*) of 14 selected mutants. In addition, kanamycin resistance encoded by the T-DNA insertion did not cosegregate with the rat phenotype in the mutant *rat17* (C.T.R. Kumar, unpublished data). The T-DNA insertion site in *rat17* is in the 3′-UTR of the *cpc* (caprice) gene. Another mutant containing a T-DNA insertion in the coding region of this gene (Wada et al., 1997) is not a *rat* mutant (C.T.R. Kumar, unpublished data). We have recovered plant DNA/

Table I. *rat mutants*

*, Mutant complemented with wild-type gene; **, attempted complementation failed; ***, kanamycin (kan) resistance does not cosegregate with rat phenotype; +, mutant scores less than 25% of wild-type; ++, mutant scores less than 33% of wild-type; +++, mutant scores less than 50% of wild-type; ++++, mutant scores more than 50% of wild-type but still a rat mutant; +++++, mutant scores at the level of wild-type for transient GUS activity; N/A, not applicable.

Mutant	Identification[a]	Collection	Tumorigenesis	Phosphinothricin (ppt) Resistance	Transient GUS	Zygosity	Resistance Marker	Gene Affected	Insertion Site
*rat1**	F	Feldmann	+	+	+	homo	kan	Arabinogalactan protein	5′-Untranslated region (UTR)
*rat3**	F	Feldmann	+	+	+++	homo	kan	Likely cell wall protein	Intergenic
*rat4**	F	Feldmann	+	+	+	homo	kan	Cellulose synthase-like protein (CslA-09)	3′-UTR
*rat5**	F	Feldmann	+	+	+++++	homo	kan	Histone H2A-1	3′-UTR
rat6	F	Feldmann	+	+	+		kan		
rat7	F	Feldmann	+	+	+		kan	Unknown protein	
rat8	F	Feldmann	+++	+++	++		kan		
rat9	F	Feldmann	+	+	+		kan	Unknown protein	
rat10	F	Feldmann	+	+	++		kan		
rat11	F	Feldmann	+	+	++		kan		
rat12	F	Feldmann	+	+	+		kan		
rat13	F	Feldmann	+	+	+		kan		
rat14	F	Feldmann	+	+	++		kan	Unknown protein	3′-UTR
rat15	F	Feldmann	+	+	+		kan		
rat16	F	Feldmann	+	+++	++		kan		
*rat17***	F	Feldmann	+	+	+++++		kan	Myb transcription factor (*cpc*)	3′-UTR
rat18	F	Feldmann	+	++	+++++		kan		
rat19	F	Feldmann	+	+	+		kan		Intergenic
rat20	F	Feldmann	+	+++	+++++		kan		
rat21	F	Feldmann	+	+	+++		kan		
rat22	F	Feldmann	+	++	++++		kan	Unknown protein	Intergenic
rat A1	F	Feldmann	+	+		homo	kan		
*rat A2**	F, R	Feldmann	+	+	+	homo	kan	phosphatase 2A (*rcn1*)	Sixth exon
rat A3	F	Feldmann	+	+		homo	kan		
rat A4	F	Feldmann	+	++++		homo	kan	Kinesin protein	First intron
rat A5	F	Feldmann	+	++++		homo	kan	Unknown protein	
rat A6	F	Feldmann	+	+		homo	kan		
*ratJ1**	F	Feldmann	+	++	+	homo	kan	Importin β-3	18th intron
ratJ2	F	Feldmann	+	+			kan	MADS box protein	Fifth intron
ratJ3	F	Feldmann	+				kan		
ratJ4	F	Feldmann	+	++			kan		
ratJ5	F	Feldmann	+	+			kan		
ratJ6	F	Feldmann	+	+			kan	3- Isopropylmalate dehydrogenase	Sixth exon
ratJ7	F	Feldmann	+	+		homo	kan	DEAD box RNA helicase	Third intron
ratJ8	F	Feldmann	+	+			kan		
ratJ9	F	Feldmann	+	+			kan	Mitochondrial chaperonin hsp60	Fourth exon
ratJ10	F	Feldmann	++	+			kan		
ratJ11	F	Feldmann	+	+			kan		
ratJ12	F	Feldmann	+	+			kan		
ratJ13	F	Feldmann	+	+			kan		
ratJ14	F	Feldmann	+	+			kan		
ratJR1	F	Feldmann	+	+			kan		
ratJR2	F	Feldmann	++	+			kan		
ratJR3	F	Feldmann	+	+			kan		
ratJR4	F	Feldmann	++	++++			kan		
ratJR5	F	Feldmann	++		+++		kan		

(*Table Continues*)

Table I. *Continued*

Mutant	Identification[a]	Collection	Tumorigenesis	Phosphinothricin (ppt) Resistance	Transient GUS	Zygosity	Resistance Marker	Gene Affected	Insertion Site
ratJR6	F	Feldmann	++++	++			kan		
ratJR7	F	Feldmann	+	+	+		kan		
ratJR8	F	Feldmann	+	++	++		kan		
ratJR9	F	Feldmann	+++	+	++++		kan		
ratJR10	F	Feldmann	++++	+	++++		kan		
ratJR11	F	Feldmann	++++	+	+		kan		
ratL1	F	Bressan	+++				ppt	Cyclin/cinnamoyl transferase	Third intron/3′-UTR
ratL2	F	Bressan	+++				ppt		
ratL3	F	Bressan	+++				ppt	ARID protein/METHF dehydrogenase	5′-UTR/Fourth exon
ratL4	F	Bressan	+++				ppt	ATP citrate lyase/glucosidase	5′-UTR
ratL5	F	Bressan	+				ppt	A-T-rich repeat region	Intergenic
ratL6	F	Bressan	++				ppt	Hypothetical protein	Exon
ratL7	F	Bressan	+				ppt	CAAT repeat region	3′-UTR
ratT2	F	Feldmann	++	++			kan		
ratT3	F	Feldmann	+++	+			kan	rac GTPase-activating protein	5′-UTR
ratT4	F	Feldmann	+	+			kan	Ethylene-responsive element binding factor	3′-UTR
*ratT5**	F	Feldmann	+	+	++	homo	kan	DREB2A	Second exon
ratT6	F	Feldmann	+	+			kan		
ratT7	F	Feldmann	+	+			kan		
*ratT8***	F	Feldmann	+	+	+	homo	kan	Receptor-like kinase	3′-UTR
ratT9	F	Feldmann	+	+			kan	Receptor-like kinase	3′-UTR
ratT10	F	Feldmann	+	+			kan	Unknown protein	3′-UTR
ratT11	F	Feldmann	+	+			kan		
ratT12	F	Feldmann	+	++			kan		
ratT13	F	Feldmann	+	+			kan	Unknown protein	5′-UTR
ratT14	F	Feldmann	++	+++			kan		
ratT15	F	Feldmann	+	+			kan	Unknown protein	5′-UTR
*ratT16**	F	Feldmann	+	+	+	homo	kan	Unknown protein	6th intron
*ratT17**	F	Feldmann	+	++	++++	homo	kan	Histone H3	Intergenic
ratT18	F	Feldmann	+	+			kan	β-Expansin	Intron
ratT19	F	Feldmann	+						
*uta1**	F	Bressan	+			homo	ppt	Voltage-dependent anion channel	First exon
uta2	F	Bressan	+			homo	ppt	F-box protein	Exon
act2-1	R	Feldmann	+	+	++	homo	kan	Actin-2 (root actin)	First intron
act7-4*	R	Feldmann	+	+	+++	homo	kan	Actin-7 (root actin)	First intron
act7-1*	R	Feldmann	+	+++	+++	homo	kan	Actin-7 (root actin)	Fourth intron
Importin α-7*	R	Feldmann	+	++	+++	homo	kan	Importin α-7	Seventh intron
ratH1	R	Feldmann	+	+	+++		kan	Unknown protein	Intergenic
cep1	R	Feldmann	+		+	homo	kan	Constitutive expression of PR1,2,5 genes	
*atrx1**	R	Feldmann	+			homo	kan	*Atrx1*	
rad5	R		+	++++	++				Point mutation
HTA2	R	Feldmann	++			homo	kan	Histone H2A-2	5′-UTR
HTA3	R	Feldmann	+			homo	kan	Histone H2A-3	3′-UTR
HTA10	R	Feldmann	++++		+++++	homo	kan	Histone H2A-10	3′-UTR
HTA11	R	Feldmann	+			homo	kan	Histone H2A-11	3′-UTR

(*Table Continues*)

Table I. *Continued*

Mutant	Identification[a]	Collection	Tumorigenesis	Phosphinothricin (ppt) Resistance	Transient GUS	Zygosity	Resistance Marker	Gene Affected	Insertion Site
HTA13	R	Feldmann	++++		+++++	homo	kan	Histone H2A-13	5′-UTR
HTB5	R	Feldmann	++			homo	kan	Histone H2B-5	5′-UTR
HTB6	R	Feldmann	++		+++++	homo	kan	Histone H2B-6	3′-UTR
HTR4/5	F, R	Feldmann	+++			homo	kan	Histone H3-4/5	Intergenic
HFO3	R	Feldmann	+			homo	kan	Histone H4-3	3′-UTR
HFO4	R	Feldmann	+		+++++	homo	kan	Histone H4-4	3′-UTR
HAT6	R	Feldmann	+	+	+++	homo	kan	Histone acetyl transferase-6	5′-UTR
HAC11	R	Feldmann	++		+++	homo	kan	Histone acetyl transferase-11	3′-UTR
HDA1	R	Feldmann	+		+++++	homo	kan	Histone deacetylase-1	Exon
HDA2	R	Feldmann	++++		+++++	homo	kan	Histone deacetylase-2	Exon
HDA6	R	Feldmann	+++			homo	kan	Histone deacetylase-6	3′-UTR
HDA9	R	Feldmann	++++			homo	kan	Histone deacetylase-9	3′-UTR
HXA1	R	Feldmann	+++		+	hetero	kan	Histone acetylase complex HXA1	5′-UTR
HXA2	R	Feldmann	+		++++		kan	Component ADA2 homolog HXA2	5′-UTR
CS2491	R	Feldmann	+			homo	kan	Disease resistance gene	
Unknown (HAT4)	R	Feldmann	+			homo	kan	Homologous to phytoene hydroxylase	
RNAi CHA 6A	R		++++				hyg	Chromatin-remodeling complex subunit 6	N/A
RNAi CHA 6C	R		++++				hyg	Chromatin-remodeling complex subunit 6	N/A
RNAi HAC8	R	Line 156 A	+			homo	hyg	Histone acetyl transferase-8	N/A
	R	Line 156 B	+			homo	hyg	Histone acetyl transferase-8	N/A
RNAi NFA2	R	Line 300 A	+++			homo	hyg	Nucleosome assembly factor A	N/A
	R	Line 422 A	++++			homo	hyg	Nucleosome assembly factor A	N/A
RNAi SGA1	R	Line 524 A	++++			homo	hyg	Chromatin-silencing group 1	N/A
	R	Line 524 B	+			homo	hyg	Chromatin-silencing group 1	N/A
RNAi BTI1	R	Line 23	+		+		ppt	Unknown protein	N/A
RNAi BTI2	R	Line 45	+		+		ppt	Unknown protein	N/A
RNAi BTI3	R	Line 16	+		+		ppt	Unknown protein	N/A
RNAi AtRAB8	R	Line 22	+		+		ppt	AtRAB8	N/A
rat4 Antisense lines	R	Line L	+++				hyg	CslA-09	N/A
	R	Line M	++++				hyg	CslA-09	N/A
	R	Line N	++++				hyg	CslA-09	N/A
Importin α-1 antisense lines	R	Line 1	+++		+++		ppt	Importin α-1	N/A
	R	Line 2	++++		++++		ppt	Importin α-1	N/A
	R	Line 3	++		++		ppt	Importin α-1	N/A
	R	Line 4	+++		++		ppt	Importin α-1	N/A

(*Table Continues*)

Table I. *Continued*

Mutant	Identification[a]	Collection	Tumorigenesis	Phosphinothricin (ppt) Resistance	Transient GUS	Zygosity	Resistance Marker	Gene Affected	Insertion Site
BTI1 antisense	R	Line 132	++	+	+		kan	Unknown protein	N/A
BTI2 antisense	R	Line 107	+	+++	+		kan	Unknown protein	N/A
RAB8 antisense	R	Line 19	+	+++	+++		kan	AtRAB8	N/A
VIP1 antisense	R	(In tobacco [*Nicotiana tabacum*])	+				kan	VirE2-interacting protein	N/A

[a] Mutant identified by forward (F) or reverse (R) genetics

T-DNA junction sequences from more than one-half of the *rat* mutants. In most cases, the T-DNA inserted outside of the predicted coding region of the gene. However, a few *rat* mutants contain T-DNA insertions within a predicted intron or a predicted exon. The paucity of T-DNA insertions in predicted exons and introns of *rat* mutants is striking and suggests that insertions in the open reading frames of these genes may be deleterious to plant survival. A propensity to recover T-DNA insertions outside protein coding regions of the gene has been noted by others (Rios et al., 2002; Szabados et al., 2002) and may reflect the tendency of the T-DNA to target A-T-rich regions of the genome for integration (Brunaud et al., 2002).

Classification of Genes Involved in the Rat Phenotype

Considering T-DNA insertion, antisense, and RNAi mutants, we have identified a wide range of genes that contribute to *A. tumefaciens*-mediated transformation. These can be classified into several general groups (Table II).

Chromatin Structure and Remodeling Genes

Based on our initial discovery that *rat5* contains a disruption of the histone H2A gene *HTA1* and that this gene is involved in T-DNA integration (Mysore et al., 2000b), we have conducted an extensive search for T-DNA insertions in all Arabidopsis core histone, histone acetyltransferase, and histone deacetylase genes. We have coupled this search with an analysis of Arabidopsis lines containing RNAi constructions directed against numerous chromatin genes (see ChromDB at http://www.chromdb.org/). *rat* mutants within this group include disruptions of five additional histone H2A genes, two histone H2B genes, two histone H3 genes, and two histone H4 genes. The *RAT5* histone H2A gene *HTA1* may encode a "replacement" histone because it is expressed in cells that are not carrying out mitotic division, although these cells may be undergoing endoreduplication (Yi et al., 2002). A T-DNA disruption between two closely spaced histone H3 genes (*HTR5* and *HTR4*) also results in a rat phenotype. *HTR4* and *HTR5* encode "replacement" histones (Chaubet et al., 1992; Chaubet-Gigot et al., 2001). These results suggest that "replacement" histones may be involved in *A. tumefaciens*-mediated transformation. T-DNA or RNAi disruptions of other chromatin modifying genes, including those that encode four histone deacetylases, five histone acetyl transferases, and three other chromatin-modifying proteins, also result in a rat phenotype. Many of these *rat* mutants remain susceptible to transient transformation, suggesting the importance of chromatin structure in T-DNA integration.

Nuclear-Targeting Genes

T-complex protein components VirD2 and VirE2 interact in yeast (*Saccharomyces cerevisiae*) with a number of Arabidopsis proteins that are involved in nuclear targeting of karyophilic proteins, including importin-α and VIP1 (Ballas and Citovsky, 1997; Tzfira and Citovsky, 2001; Tzfira et al., 2001; S. Bhattacharjee and S.B. Gelvin, unpublished data). Disruption of importin-α7 and importin-β3 (*ratJ1*) by T-DNA insertion and importin-α1 and VIP1 by antisense inhibition result in a rat phenotype. These results emphasize the importance of nuclear transport of the T-complex as a key step of the *A. tumefaciens*-mediated transformation process.

Cytoskeleton Genes

Mutations in two root-expressed actins (actin-2 and actin-7; McKinney et al., 1995; Kandasamy et al., 2001), but not the pollen-expressed actin-12, result in a rat phenotype. A mutant with a T-DNA insertion in a kinesin gene is also a *rat* mutant. However, the *bot1* mutant that has altered cortical microtubule organization (Bichet et al., 2001) does not show a rat phe-

Table II. *Steps of the transformation process putatively disrupted in selected Arabidopsis rat mutants*

+, Mutant scores less than 25% of the wild-type; ++, mutant scores less than 33% of the wild-type; +++, mutant scores less than 50% of the wild-type; ++++, mutant scores more than 50% of the wild-type but still a *rat* mutant; +++++, mutant scores at the level of wild-type for transient GUS activity; *, mutant has been complemented with the wild-type gene; ND, not determined.

Mutant	Tumorigenesis	Transient GUS	Gene Affected
Bacterial attachment/T-DNA transfer			
*rat1**	+	+	Arabinogalactan protein
*rat3**	+	+++	Likely cell wall protein
*rat4**	+	+	AtCslA-09
ratT18	+	ND	β-Expansin
Antisense *rat4*	+++	ND	AtCslA-09
Antisense F9	++	+	Unknown protein
Antisense F8	+	+	Unknown protein
Antisense RAB8	+	+++	AtRAB8
RNAi BTI1	+	+	Unknown protein
RNAi BTI2	+	+	Unknown protein
RNAi BTI3	+	+	Unknown protein
RNAi AtRAB8	+	+	AtRAB8
Cytoplasmic trafficking/cytoskeleton			
act2-1	+	++	Actin-2
act7-4*	+	+++	Actin-7
act7-1*	+	+++	Actin-7
rat A4	+	ND	Kinesin protein
Nuclear targeting			
*ratJ1**	+	+	Importin β-3
Importin α-7*	+	+++	Importin α-7
Antisense importin α-1	++/+++	++/+++	Importin α-1
T-DNA integration/chromatin structure and remodeling			
*rat5**	+	++++	Histone H2A-1
HTA2	++	ND	Histone H2A-2
HTA3	+	ND	Histone H2A-3
HTA10	++++	+++++	Histone H2A-10
HTA11	+	ND	Histone H2A-11
HTA13	++++	+++++	Histone H2A-13
HTB5	++	ND	Histone H2B-5
HTB6	++	+++++	Histone H2B-6
HTR4/5	+++	ND	Histone H3-4/5
HFO3	+	ND	Histone H4-3
HFO4	+	+++++	Histone H4-4
HDA1	+	+++++	Histone deacetylase-1
HDA2	++++	+++++	Histone deacetylase-2
HDA6	+++	ND	Histone deacetylase-6
HDA9	++++	ND	Histone deacetylase-9
HAT6	+	+++	Histone acetyl transferase-6
HAC11	++	+	Histone acetyl transferase-11
HXA1	+++	+	Histone acetylase complex HXA1
HXA2	+	++++	Histone acetylase complex HXA2
RNAi CHA6	++++	ND	Chromatin-remodeling complex subunit 6
RNAi HAC 8-1	+	ND	Histone acetyl transferase-8
RNAi NFA2-1	+++	ND	Nucleosome assembly factor A
RNAi SGA1	+	ND	Chromatin-silencing group 1

notype. VirD2 and VirE2 proteins interact with polymerized actin in vitro, and pharmacological inhibitors of actin cytoskeleton structure or the myosin motor reversibly inhibit transformation of tobacco BY-2 cells (P. Rao, M. Duckely, B. Hohn, and S.B. Gelvin, unpublished data). These results suggest a role for the plant cytoskeleton in the transformation process, possibly by mediating cytoplasmic trafficking of the T-complex.

Cell Wall Structural and Metabolism Genes

Attachment of *A. tumefaciens* to plant cells is required for efficient transformation (Matthysse, 1987). Several *rat* mutants contain T-DNA insertions in or near genes implicated in cell wall synthesis or modification. *rat1* contains a T-DNA insertion in the promoter region of a gene encoding an arabinogalactan protein, and *A. tumefaciens* do not attach well to roots

of this mutant (Nam et al., 1999; Y.M. Gaspar, J. Nam, C.J. Schultz, L.-Y. Lee, P. Gilson, S.B. Gelvin, and A. Bacic, unpublished data). Arabinogalactan proteins are important for transformation. Incubation of Arabidopsis roots with β-glucosyl Yariv reagent, a chemical that binds arabinogalactan proteins, inhibits transformation (J. Nam and S.B. Gelvin, unpublished data). *rat4* contains a T-DNA insertion that disrupts transcription of a cellulose synthase-like gene (*cslA-09*). This mutant shows a decreased number of lateral roots (Y. Zhu and S.B. Gelvin, unpublished data). A β-expansin mutant was also isolated that shows a strong *rat* phenotype. Finally, we have identified, using a yeast two-hybrid approach, a number of "unknown" Arabidopsis proteins that may serve as putative receptors for the *A. tumefaciens* T-pilus. These proteins localize to the periphery of the plant cell. Antisense and RNAi inhibition of expression of the genes that encode these proteins results in a rat phenotype (H.-H. Hwang and S.B. Gelvin, unpublished data).

Other RAT *Genes*

Numerous other mutants were obtained containing disruptions in genes likely involved in signal transduction processes, including a receptor-like protein kinase (*ratT8* and *ratT9*) and a type 2A phosphoprotein phosphatase (*ratA2*). Other *rat* mutants contain T-DNA insertions in genes whose products may be involved in the process of gene expression or protein function. These include a DEAD box RNA helicase (*ratJ7*), a MADS box protein (*ratJ2*), a stress-related DREB2A transcription factor (*ratT5*), and an F-box protein (*uta2*). DNA sequence analysis of plant DNA/T-DNA junctions from a large number of *rat* mutants identified proteins of unknown function, or "hypothetical" proteins.

CONCLUSIONS

Using a combination of forward and several reverse genetic strategies, we have identified 126 Arabidopsis *rat* mutants. Many of these mutants can be transiently transformed, suggesting that in these mutants the step of T-DNA integration is specifically disrupted. Other mutants show defects in bacterial attachment to roots. Although we could genetically complement 13 of 14 selected mutants to transformation proficiency with the corresponding wild-type gene, we have not attempted complementation of the majority of mutants. Thus, the reader should be aware that we have not proven that disruption of many genes by T-DNA insertion is responsible for the rat phenotype. We continue to characterize these *rat* mutants and search for additional *rat* mutants. We shall periodically update the results of these activities (http://www.bio.purdue.edu/courses/gelvinweb/gelvin.html). All *rat* mutants are available for further investigation (please contact Stanton B. Gelvin at gelvin@bilbo.bio.purdue.edu).

MATERIALS AND METHODS

Agrobacterium tumefaciens Strains and Culture Conditions

All *A. tumefaciens* strains were cultured in liquid Yeast Extract-Peptone medium (Lichtenstein and Draper, 1986) containing the appropriate antibiotics (10 μg mL^{-1} rifampicin and 25 μg mL^{-1} kanamycin). Crown gall tumorigenesis assays were conducted using *A. tumefaciens* A208 (Sciaky et al., 1978), which incites large, green teratomas on the roots of Arabidopsis ecotype Ws. *A. tumefaciens* At872 (Nam et al., 1999), containing a plant-active *bar* gene on the binary vector pCAS1, was used to incite ppt-resistant calli. *A. tumefaciens* At849 (Nam et al., 1999) contains the binary vector pBISN1 (Narasimhulu et al., 1996). pBISN1 contains a plant-active *nptII* gene and a *gusA*-intron gene under the control of a "super-promoter" (Ni et al., 1995). The intron in the *gusA* gene prevents expression of GUS activity in bacteria (Liu et al., 1992). *A. tumefaciens* At849 was used to incite kanamycin-resistant calli and to monitor transient GUS expression in inoculated root segments.

Root Transformation Assays

We have previously described seed sterilization and germination, plant growth, preparation of *A. tumefaciens*, and in vitro root inoculation procedures (Nam et al., 1997, 1998, 1999; Mysore et al., 2000b; Yi et al., 2002). In brief, surface-sterilized Arabidopsis seeds were placed on Gamborg's B5 medium (Gibco-BRL, Gaithersburg, MD) solidified with 0.75% (w/v) Bactoagar (Difco, Detroit) and containing the appropriate selective agent (50 μg mL^{-1} kan or 10 μg mL^{-1} ppt). After incubation at 4°C for 2 d, the plates were incubated under a 16-h-light/8-h-dark photoperiod at 25°C for 7 to 10 d. Individual seedlings were transferred into baby food jars containing solidified B5 medium lacking a selective agent and grown for 7 to 10 d. Roots were cut into 3- to 5-mm segments, and bundles of roots from an individual plant were inoculated with the appropriate *A. tumefaciens* strain. After 2 d, the root bundles were separated into individual segments and transferred to solidified medium containing 100 μg mL^{-1} timentin to kill the bacteria and the appropriate agent to select for transformation. We used Murashige and Skoog medium (Gibco-BRL) lacking phytohormones to select for crown gall tumors and callus-inducing medium (Nam et al., 1997) containing either kan (50 μg mL^{-1}) or ppt (10 μg mL^{-1}) to select for antibiotic- or herbicide-resistant calli, respectively. For GUS activity assays, root segments were placed on solidified callus-inducing medium for 4 to 6 d, after which they were stained with 5-bromo-4-chloro-3-indolyl glucuronide (Jefferson et al., 1987).

PCR-Based Reverse Genetic Approach to Identify T-DNA Insertions in Genes

We used a PCR-based approach similar to that described by Krysan et al. (1996) to identify Arabidopsis (ecotype Ws) mutants containing a T-DNA insertion in or near a gene of interest. Pooled samples of DNA from 1,000, 100, and 20 plants from the Feldmann T-DNA insertion library (Feldmann and Marks, 1987; Forsthoefel et al., 1992) were successively assayed for insertions, followed by assay of individual plants from the pool of 20 mutant plants. The zygosity of a particular allele was determined using forward and reverse primers specific to the particular gene and one primer specific to a particular gene in combination with either a T-DNA left or right border primer. T-DNA primer sequences were: left border, 5′-GATGCACTCGAAATCAGCCAATTTTAGAC-3′; and right border, 5′-TCCTTCAATCGTTGCGGTTCTGTCAGTTC-3′. To identify T-DNA insertions in or near large genes, primer sets were designed approximately every 2 kb along the gene, including primers reading "out" from the 5′ and 3′ ends of the open reading frame. PCR was carried out using 0.5 units of ExTaq (Takara, Shiga, Japan) DNA polymerase with robocyclers (Stratagene, La Jolla, CA) using the following amplification conditions: 95°C for 5 min, 30 to 36 cycles at 94°C for 40 to 60 s, 56°C to 60°C (depending upon the primer melting temperature) for 1 min, 72°C for 3 min, 72°C for 10 min, and 4°C hold. We

used 0.24 μM of each primer, 0.2 mM of each dNTP, and either 100 ng of DNA (for screening super-pools of 1,000 plants) or 20 ng of DNA (for screening pools of 100, 20, or individual plants) in a 50-μL final reaction volume.

Antisense and RNAi Reverse Genetic Approaches

To construct plasmids to express antisense versions of a given gene, large portions of the cDNA of that gene were cloned into the T-DNA binary vector pE1546 under the control of an enhanced cauliflower mosaic virus 35S promoter in an antisense orientation. pE1546 contains a plant-active *hpt* gene for selection of hygromycin-resistant plants. The resulting construction was introduced into *A. tumefaciens* GV3101 (Koncz and Schell, 1986) by electroporation, and this strain was used to transform the appropriate Arabidopsis mutant line using a "flower dip" technique (Clough and Bent, 1998). Although *rat* mutants are highly recalcitrant to root transformation, they are easily transformed by "flower vacuum infiltration" or "flower dip" techniques (Mysore et al., 2000a). Plants containing the antisense gene were identified by selection on 20 μg mL^{-1} hygromycin. To construct plasmids for RNAi experiments, large portions of the cDNA encoding the open reading frame of the gene of interest were cloned in both sense and antisense orientation into the T-DNA binary vectors pFGC1008 (hygromycin selection in plants) or pFGC5941 (ppt selection in plants; http://www.chromdb.org/). The resulting plasmids were introduced into GV3101, and the *A. tumefaciens* strain was used to transform the relevant mutant plant as described above.

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